MAJOR ARTICLE

Presence of Nonhemolytic Pneumolysin in Serotypes of *Streptococcus pneumoniae* Associated with Disease Outbreaks

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Pneumolysin is an important virulence factor of the human pathogen *Streptococcus pneumoniae*. Sequence analysis of the *ply* gene from 121 clinical isolates of *S. pneumoniae* uncovered a number of alleles. Twenty-two strains were chosen for further analysis, and 14 protein alleles were discovered. Five of these had been reported previously, and the remaining 9 were novel. Cell lysates were used to determine the specific hemolytic activities of the pneumolysin proteins. Six strains showed no hemolytic activity, and the remaining 16 were hemolytic, to varying degrees. We report that the nonhemolytic allele reported previously in serotype 1, sequence type (ST) 306 isolates is also present in a number of pneumococcal isolates of serotype 8 that belong to the ST53 lineage. Serotype 1 and 8 pneumococci are known to be associated with outbreaks of invasive disease. The nonhemolytic pneumolysin allele is therefore associated with the dominant clones of outbreak-associated serotypes of *S. pneumoniae*.

Pneumolysin is a 53-kDa protein produced by most clinical isolates of *Streptococcus pneumoniae* [1, 2] and is an important virulence factor of the organism, which is a causative agent of pneumonia, bacteremia, meningitis, otitis media, and conjunctivitis in humans. Pneumolysin is a cholesterol-dependent pore-forming toxin and has lytic effects on many cell types. In addition to cell lysis, pneumolysin has a number of effects on host systems when present at sublytic levels [3–9], including complement activation in the absence of anti-pneumolysin antibodies and induction of proinflammatory mediators. Complement activation is independent of the hemolytic activity of the toxin. Pneumolysin has recently been shown to stimulate mouse macrophages to produce tumor necrosis factor–α and interleukin-6 via interaction with Toll-like receptor (TLR) 4 and myeloid differentiation marker 88. This is also independent of hemolytic activity [10]. Pneumolysin is produced by the majority of pneumococci regardless of serotype [1, 11], and toxoids are being considered as vaccine candidates [12–14], either alone or as a carrier protein for the pneumococcal polysaccharides in current vaccines.

The amino acid sequence of pneumolysin is thought to be relatively conserved throughout all pneumococcal serotypes with little variance over time and geographic distance [15]. However, Lock et al. [16] have reported that 2 isolates of serogroups 7 and 8 possess a threonine

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to isoleucine substitution at position 172. Both alleles also contained a 2-aa deletion of valine 270 and lysine 271. Only the T172I substitution affected the hemolytic activity of pneumolysin. Recently, data from our laboratory have shown that serotype 1, sequence type (ST) 306 pneumococci produce a nonhemolytic pneumolysin [17]. This allele contains all the mutations reported for the serotype 8 strain by Lock et al. as well as a tyrosine to histidine substitution at position 150 [17].

Serotype 1 pneumococci are unusual in being associated with disease outbreaks [18–22]. The majority are linked to conditions of overcrowding or alcoholism. In addition, serotype 1 pneumococci have a high attack rate and are rarely isolated from nasopharyngeal carriage [23, 24]. Serotype 8 pneumococci have also been associated with linked cases of disease [25].

Nonhemolytic pneumolysin has been previously observed in serotype 1, ST306 isolates in our laboratory, and, hence, the aim of the present study was to assess pneumolysin variation and determine the distribution of pneumolysin alleles—in particular the nonhemolytic allele—in the serotype 1 pneumococcal population. Because Lock et al. [16] have previously described pneumolysin with reduced hemolytic activity in certain serotype 8 pneumococci, a similar analysis of pneumolysin diversity within the serotype 8 population was undertaken.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and sample preparation. A list of strains used in the present study can be found in table 1. Pneumococcal strains were obtained from the Scottish Meningococcal and Pneumococcal Reference Laboratory (SMPRL). Pneumococci are received by SMPRL via an enhanced surveillance scheme whereby >90% of isolates from persons with invasive disease are received. These are serotyped and subjected to multilocus sequence typing (MLST). Strains were also provided by R.C.G., A.B.B., and G.P. SMPRL isolates were chosen to represent a wide range of serotypes, including those not commonly isolated from persons with invasive disease, and to be well distributed with respect to geographical location within Scotland, disease type, and age of patient. Strains donated by A.B.B. were representative of those used in a study of distribution and diversity of serotype 1 pneumococci [23]. Strains from Ghana represented the 3 most prevalent clones observed [18]. Strains were grown in brain-heart infusion (BHI) broth (Oxoid) and stored as frozen stocks on Protect beads (Technical Service Consultants). Cells were grown in 50 mL of BHI broth until midlogarithmic phase (OD_{600} between 0.55 and 0.65). Cell lysates were prepared from 40 mL of culture, as described elsewhere [17].

Measurement of pneumolysin activity. Pneumolysin concentration was determined using an ELISA, as described elsewhere [17]. Hemolytic assays were performed as described elsewhere [28], with modifications. First, 50 μL of 10 mmol/L dithiothreitol was added before the addition of erythrocytes. This was followed by a 30-min incubation at 37°C. At the end of the assay, 50 μL of PBS was added, and plates were centrifuged at 179 *g* in a bench-top centrifuge (model 4K15; Sigma) for 1 min. One hundred microliters of supernatant was removed, and the absorbance was measured at 540 nm.

The percentage of hemolysis and the log pneumolysin concentration were plotted against each other. The pneumolysin concentration required to produce 50% lysis of erythrocytes was determined. The reciprocal of this value gave the specific activity, in hemolytic units (HU) per milligram, of the pneumolysin. Western blotting was done as described elsewhere [17].

DNA sequencing. Pneumococcal *ply* genes were amplified by polymerase chain reaction (PCR) from gDNA preparations using platinum *Taq* polymerase (Invitrogen) and primers 27R and 27S, listed in table 2. PCR products were cleaned using the AMPure PCR purification method (Agencourt Bioscience). DNA sequencing was performed using Big Dye Terminator cycle sequencing reagents (version 1.1; Applied Biosystems) and a MegaBace 1000 DNA sequencer (Amersham). All primers are shown in table 2. Full *ply* sequences were deposited in GenBank under accession numbers EF413923–EF413960.

MLST analysis. e-BURST (version 2; http://www.mlst.net/) was used to illustrate relationships between isolates listed in the pneumococcal MLST database.

RESULTS

Sequence variation among pneumolysin genes. Partial DNA sequences (data not shown) of *ply* genes from 121 strains were aligned using the ClustalX interface (http://www.ebi.ac.uk/clustalw/). Twenty-two isolates were chosen for further analysis, because the others had pneumolysin alleles closely related to the wild-type D39 strain. Variation in the DNA sequence occurred at 37 positions in the pneumolysin of these isolates. The DNA alleles were translated into protein sequences, and these sequences were then aligned using ClustalX. Amino acid sequences showed variation at 15 positions, and 2 alleles contained regions of insertion (figure 1). Five of the pneumolysin alleles we identified had been previously reported [16, 17, 26, 27].

Alleles were aligned and numbered with the wild-type allele (to which others were compared) being allele 1 (figure 1). This allele is expressed by strain D39 and was the first pneumolysin gene to be sequenced [26]. This protein had a specific activity of 4.13×10^5 HU/mg. Allele 2, present in strain TIGR4, had similar levels of hemolytic activity. Along with allele 1, allele 2 was the most common allele apparent in our initial screen. Alleles 7, 9, 11, and 13 had similar specific activity and will not be discussed further here.

Allele 3 was discovered in 2 serotype 8 isolates and is identical

Table 1. Strains used in the present study, by serotype and sequence type (ST) and with pneumolysin allele and source detailed.

Strain	Serotype	ST	Pneumolysin allele	Source or reference
D39	2	128	1	Walker et al. [26]
TIGR4	4	205	2	Tettelin et al. [27]
S1_46	1	217	1	Brueggemann and Spratt [23]
P1041	1	217	2	Leimkugel et al. [18]
01_2696	1	227	2	Kirkham et al. [17]
S1_2	1	227	2	Brueggemann and Spratt [23]
00-3645	1	227	4	Kirkham et al. [17]
04-2055	1	228	5	SMPRL
S1-11	1	228	14	Brueggemann and Spratt [23]
INV1871	1	300	2	Brueggemann and Spratt [23]
P1039	1	303	1	Leimkugel et al. [18]
S1_8	1	303	2	Brueggemann and Spratt [23]
S1_4	1	304	2	Brueggemann and Spratt [23]
S1_30	1	305	2	Brueggemann and Spratt [23]
S1_3	1	306	5	Brueggemann and Spratt [23]
01-1956	1	306	5	Kirkham et al. [17]
S1_71	1	611	2	Brueggemann and Spratt [23]
S1_45	1	612	2	Brueggemann and Spratt [23]
P1021	1	612	2	Leimkugel et al. [18]
S1_125	1	613	1	Brueggemann and Spratt [23]
S1_126	1	614	1	Brueggemann and Spratt [23]
NCTC 7465	1	615	2	Brueggemann and Spratt [23]
S1_102	1	616	2	Brueggemann and Spratt [23]
S1_38	1	617	5	Brueggemann and Spratt [23]
S1_99	1	618	2	Brueggemann and Spratt [23]
03_5343	1	1239	5[a]	SMPRL
03_5340	1	1310	5[a]	SMPRL
04_1259	1	1311	5[a]	SMPRL
04_1837	1	1346	5[a]	SMPRL
04_2889	1	1597	5[a]	SMPRL
05_1635	1	1809	5[a]	SMPRL
05_1934	1	1882	5[a]	SMPRL
01_2117	1	2126	5[a]	SMPRL
96-5878	2	74	6	SMPRL
00-2328	6A	813	13	SMPRL
01-3862	7F	191	10	SMPRL
02-2744	7F	191	10	SMPRL
01-2884	8	53	5	SMPRL
2PN00495	8	404	3	Robert George (HPA)
01-1204	8	578	5	SMPRL
03_2331	8	835	5[a]	SMPRL
H040920498	8	944	3	Robert George (HPA)
03_2620	8	1110	5[a]	SMPRL
H034900039	8	1722	5[a]	Robert George (HPA)
00-1153	9V	156	9	SMPRL
01-2513	18C	818	12	SMPRL
01-2914	20	591	11	SMPRL
01-2866	23F	40	8	SMPRL
01-4296	27	571	10	SMPRL
01-1199	NT	577	5	SMPRL
02-3013	NT	448	7	SMPRL

NOTE. HPA, Health Protection Agency; SMPRL, Scottish Meningococcal and Pneumococcal Reference Laboratory.

[a] The *ply* gene was partially sequenced to determine the pneumolysin allele.

Table 2. Primers used in DNA amplification and sequencing of *ply*.

Primer	DNA sequence (5′→3′)
27R	CTTGGCTACGATATTGGC
27S	TACTTGTCCAACCACGG
9Y	CGGGATCCGGCAAATAAAGCA
4T	GTTGATCGTGCTCCGATGA
4U	TATACAGTCAGCGTAGACGC
4V	CAATACAGAAGTGAAGGCGG
4W	GATCATCAAGGTAAGGAAGTC
27T	ATAAGTCATCGGAGCACG
15E	TCCAACTTGAGATAGACTTGGCGCCC
5U	GCTGTAACCTTAGTCTC
15C	GGAGGTAGAAGATGGCAAATAAAGC
52R	GAATTCCCTGTCTTTTCAAAGTC
PlyIntRev	GGCAAGCCTGGATGATCTGCTG

to the Ply8 allele found in this serotype by Lock et al. [16]. This allele was shown to have reduced hemolytic activity and reduced mobility on SDS-PAGE gel (data not shown), which agreed with the findings of Lock et al.

Allele 4, which contains an 8 aa insertion/duplication at position 415, has been previously reported [17]. This allele was found in a serotype 1, ST227 isolate and showed reduced hemolytic activity, possibly due to insolubility of the protein [17].

Allele 5 was reported initially by Kirkham et al. [17] in serotype 1, ST306 isolates. Here, we report this allele in serotype 8 clones of ST53 and related clones as well as in a nontypeable ST577 isolate. Allele 5 is similar in sequence to allele 3 but has an extra mutation at position 150. These alleles showed similarly reduced mobility on SDS-PAGE gel. However, allele 5, unlike allele 3, produces a nonhemolytic protein.

Allele 6, found in a serotype 2, ST74 isolate, was also similar to allele 3 but had a further S167F mutation. This allele had reduced hemolytic activity, resulting in a specific activity similar to allele 3.

Both alleles 8 and 10 showed reduced hemolytic activity. However, although allele 10 shares a number of mutations with the nonhemolytic allele 5, allele 8 has only a single mutation (A273D) compared with wild type. Allele 10 also shares with alleles 3 and 5 reduced mobility on SDS-PAGE gel.

Allele 12 was present in a serotype 18C, ST818 isolate and was not recognized by ELISA using the monoclonal antibody PLY-7 [29], which recognizes amino acid positions 401–407 in pneumolysin. Allele 12 is hemolytic, and Western blots using polyclonal antibodies confirm that pneumolysin is expressed. Nonrecognition using PLY-7 was also recently reported for a number of alleles of the closely related cholesterol-dependent cytolysin (CDC) mitilysin, although other alleles of this toxin were recognized [28].

Allele 14 was discovered in serotype 1, ST228 strain S1-11, isolated in Spain [23]. This allele is nonhemolytic and possesses an insertion of 871 bp at amino acid position 142 in pneumolysin. Sequencing revealed the presence of the mobile genetic element IS1515 [30] in a pneumolysin allele 2 background. This is the first demonstration of a clinical isolate of *S. pneumoniae* not expressing pneumolysin. A second ST228 isolate from invasive pneumococcal disease (IPD) in Scotland possessed allele 5 and no insertion.

eBURST analysis of the serotype 1 pneumococcal population. A search of the MLST database for serotype 1 pneumococci revealed 51 different STs of serotype 1. eBURST revealed the presence of 4 major clusters (figure 2). These clusters corresponded to the 3 geographic lineages described by Brueggemann and Spratt [23]. Lineage A, representing serotype 1 pneumococci from Europe and North America, is split into 2 clonal groups by eBURST. The predicted founders of these complexes are ST306 and ST305. ST217 is the predicted founder of lineage B, representing serotype 1 pneumococci from Africa and Israel, and is also the predicted overall founder of the serotype 1 population. ST2296 is the predicted founder of lineage C. At the time of the study by Brueggemann and Spratt [23], the majority of isolates in lineage C were from Chile. However, this lineage now contains isolates from a wider range of geographical locations.

Distribution of pneumolysin alleles within serotype 1 pneumococci distinguished by ST. Because pneumolysin allele 5 has been shown to be present in all serotype 1, ST306 pneumococcal isolates tested [17], it was of interest to determine the distribution of this allele within the serotype 1 population. Initially, *ply* genes from each of the 16 clones identified by Brueggemann and Spratt [23] were fully sequenced. Nine of these 16 strains were found to have allele 2, whereas 4 possessed allele 1, 2 had the nonhemolytic allele 5, and 1 had allele 14. The 2 isolates possessing allele 5 were clones of ST306 and ST617, and the strain possessing allele 14 was ST228. This is the first demonstration of allele 5 in serotype 1 strains other than ST306. Figure 2 shows that ST306, ST617, and ST228 are all present in lineage A. Because both hemolytic and nonhemolytic alleles were present in this lineage, we obtained 8 more lineage A isolates from SMPRL (figure 2). Partial sequencing of these isolates was used to determine the pneumolysin allele identity. This revealed that all single-locus variants of ST306 tested in lineage A possess the nonhemolytic allele 5 as well as 2 double-locus variants, for a total of 9 serotype 1 STs shown to possess allele 5. The *ply* genes from 3 serotype 1 strains representing the dominant STs from recent meningitis outbreaks in Ghana (ST217, ST303, and ST612) [18] were also sequenced. These STs were also present in the data set of Brueggemann and Spratt [23]. ST217, the predicted founder of lineage B, is associated with both pneumolysin alleles 1 and 2. Similarly, in lineage A ST228 is associated with 2 pneumolysin alleles (alleles 5 and 14), as is ST227 (alleles 2 and 4). This is

					Amino acid position																	
					14	136	142	150	154	167	172	224	260	265	267	270	271	273	380	402	415
Strain no.	Serotype	ST	Allele	Specific activty	N	Q		Y	T	S	T	K	E	A	I	V	K	A	D	Q	
D39	2	128	1	4.13×10^5																	
TIGR4	4	205	2	4.74×10^5															N		
01-2696	1	227	2	4.20×10^5															N		
2PN00495	8	404	3	6.50×10^3							I	R		S		DEL	DEL				
H040920498	8	944	3	7.43×10^3							I	R		S		DEL	DEL				
00-3645	1	227	4	–															N		INS
01-2884	8	53	5	0				H			I	R		S		DEL	DEL				
01-1204	8	578	5	0				H			I	R		S		DEL	DEL				
01-1956	1	306	5	0				H			I	R		S		DEL	DEL				
01-1199	NT	577	5	0				H			I	R		S		DEL	DEL				
04-2055	1	228	5	0				H			I	R		S		DEL	DEL				
96-5878	2	74	6	3.72×10^3						F	I	R		S		DEL	DEL				
02-3013	NT	448	7	2.95×10^5	D							R									
01-2866	23F	40	8	9.09×10^4														D			
00-1153	9V	156	9	3.14×10^5											M						
01-3862	7F	191	10	1.01×10^5					M					S		DEL	DEL		N		
02-2744	7F	191	10	9.77×10^4					M					S		DEL	DEL		N		
01-4296	27	571	10	1.36×10^5					M					S		DEL	DEL		N		
01-2914	20	591	11	5.95×10^5		K													N		
01-2513	18C	818	12	–															N	E	
00-2328	6A	813	13	3.09×10^5									D						N		
S1-11	1	228	14	–			INS												N		

Figure 1. Pneumolysin alleles determined by full sequencing of *ply* genes from 22 isolates. The nos. in the first row refer to amino acid positions, with the identity of the wild-type amino acid shown in the second row. The mutated amino acids present in specific strains are highlighted in black below the corresponding wild-type amino acid. Strains highlighted in black are nonhemolytic and as such have a specific activity of 0. Insertion regions (INS) are highlighted in gray. Deletion of an amino acid is represented by the abbreviation DEL. Specific activity is shown in hemolytic units per milligram. The lysate of strain 01-2513 (allele 12) was hemolytic but was not recognized by the monoclonal antibody PLY-7; therefore, no specific activity was determined. The lysate of strain 00-3645 (allele 4) was hemolytic but at a level that did not allow calculation of specific activity. ST, sequence type.

the first demonstration of intra-ST amino acid variation within pneumolysin. Results of hemolytic assays done on lysates of all strains sequenced were in agreement with the hemolytic profiles previously observed for these alleles.

Distribution of pneumolysin alleles within serotype 8 pneumococci distinguished by ST. Having observed pneumolysin allele 5 in 2 serotype 8 strains, an eBURST diagram was created for serotype 8 strains present in the MLST database, and isolates of this serotype available from SMPRL were partially sequenced to determine pneumolysin allele identity. eBURST identified ST53 as the predicted founder clone of serotype 8 pneumococci. This clone had pneumolysin allele 5. The 4 other STs available from the main clonal complex also had this pneumolysin allele (figure 3). There were 2 strains that possessed allele 3, identical to the Ply8 allele identified in serotype 8 pneumococci by Lock et al. [16]. These clones were predicted founders of the 2 other main clonal complexes shown by this eBURST diagram (figure 3).

Prevalence of serotype 8–related IPD in Scotland. To examine whether ST53 had been undergoing clonal expansion similar to that detailed for ST306 by Kirkham et al. [17], information from SMPRL was used to determine the prevalence of serotype 8–related IPD during the last 4 years in Scotland. The prevalence of ST53-related IPD within this serotype was also assessed. Serotype 8–related IPD has not been increasing significantly in Scotland. Approximately 5% of IPD isolates were serotype 8 in 2002–2005. ST53 is already established as the dominant clone within the Scottish serotype 8 population, representing >80% of serotype 8 isolates from 2002–2005 (data not shown).

DISCUSSION

Fourteen pneumolysin alleles were identified in this study. Previously, pneumolysin was considered to be a well-conserved protein [15]; however, 6 pneumolysin alleles had been previously reported. Here, we report the presence of 9 more novel pneumolysin alleles. The existence of at least 15 pneumolysin proteins shows that variation at the amino acid level is greater than previously thought. Pneumolysin shows a sequence variation of 3.3%. This is slightly higher than pneumococcal housekeeping genes, which show levels of sequence divergence ranging from 1% to 2% [31]. Large amounts of variation have been observed in pneumococcal surface proteins (e.g., PspC [32, 33] and NanA [34, 35]) and in antibiotic-resistance determinants, such as penicillin-binding protein genes. Variation in these proteins is thought to be due to evolutionary pressure from the host immune system or from antibiotic use [36, 37]. However, the selective evolutionary pressure on pneumolysin is unknown. The allelic variation observed here results in the expression of nonhemolytic pneumolysin in certain clonal groups of pneumococci, namely lineages of serotype 1 and serotype 8 pneumococci. Nonhemolytic pneumolysin was also observed in a nontypeable strain related by ST to clones of serotype 8 (allele 5). Because the nontypeable strain shares both pneumolysin and housekeeping alleles with serotype 8 pneu-

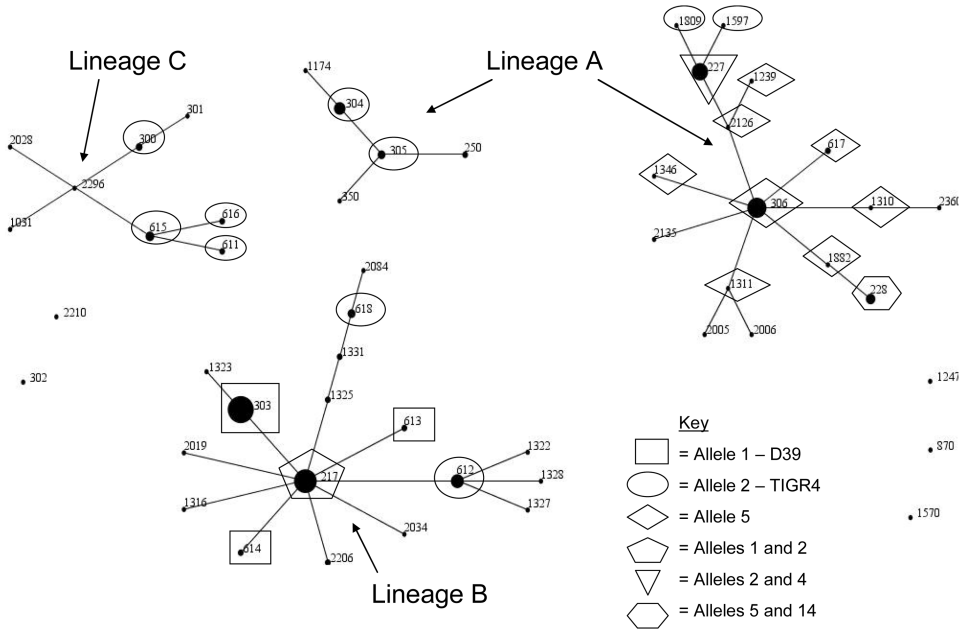

Figure 2. eBURST diagram showing the relationship between serotype 1 clones of *Streptococcus pneumoniae* as well as the distribution of pneumolysin alleles within the serotype 1 pneumococcal population.

mococci, it is possible that the isolate has serotype 8 capsule genes that are not expressed or that there is partial or full deletion of capsular genes. Given that pneumolysin alleles often correlate with the ST and the serotype of a pneumococcus, we hypothesize that the pressure to maintain alternative pneumolysin alleles stems from the pneumococcal genome itself, suggesting that certain pneumolysin alleles will be maintained only when present alongside a certain set of other pneumococcal proteins and that all of the proteins present in the set are required to work in concert to ensure the survival of the clone.

PLY-7 is currently used in an ELISA in many laboratories to determine presence or absence of pneumolysin [38]. However, the discovery of allele 12 indicates that there may be instances of false-negative results in such tests. The detection of the related CDC mitilysin by use of this antibody indicates that false-positive results are also possible [28]. Therefore, care should be taken when interpreting results obtained using this antibody as a diagnostic or therapeutic tool. Allele 12 was found in a serotype 18C, ST818 isolate. This serotype is included in the PCV7 pneumococcal conjugate vaccine; however, this isolate is the only representative of this ST in the MLST database. When eBURST was used to analyze all strains of serogroup 18/serotype 18C, ST818 was not found to be part of the major serotype 18C clonal complex.

Isolate S1-11, possessing pneumolysin allele 14, was shown to be negative for expression of pneumolysin. This result is of interest, because S1-11 was isolated from a patient with pneumonia, implying disease-causing ability without pneumolysin expression. S1-11 represents the first demonstration of a pneumococcal clinical isolate that fails to produce pneumolysin. The insertion of 871 bp present in this allele encodes IS1515, a functional insertion sequence present predominantly in serotype 1 pneumococci [39]. Because IS1515 contains internal promoter regions, expression and therefore excision of the insertion sequence may still be possible. S1-11 is a blood isolate, demonstrating the ability to progress from the lungs into the blood. IS1515 is an active mobile element that is capable of excision and insertion elsewhere in the genome. It is therefore possible that this strain produced a functional pneumolysin at the time the isolate established disease.

Distribution of pneumolysin allele 5 within the serotype 1 pneumococcal population is not confined to ST306 itself, although it is found only in the ST306 clonal complex. There appears to be a split in pneumolysin identity in lineage A. Although this correlates with ST to some extent, pneumolysin allele 2 is found in both branches. The 2 alleles are unrelated, varying by 17 nt that results in 7 aa changes. The 2 branches of lineage A are linked, as their founders are triple-locus variants of one another.

Pneumolysin allele 5 is also present in serotype 8 pneumococci. This allele is present only in the main clonal complex of this serotype (the ST53 complex). In contrast to SLVs of ST306 present in the MLST database, which are solely Scottish isolates, the SLVs of ST53 submitted to the database are from varying geographical locations. The prevalence data presented showed that ST53 is already the dominant clone in serotype 8–related IPD in Scotland. This is in contrast to published data on ST306

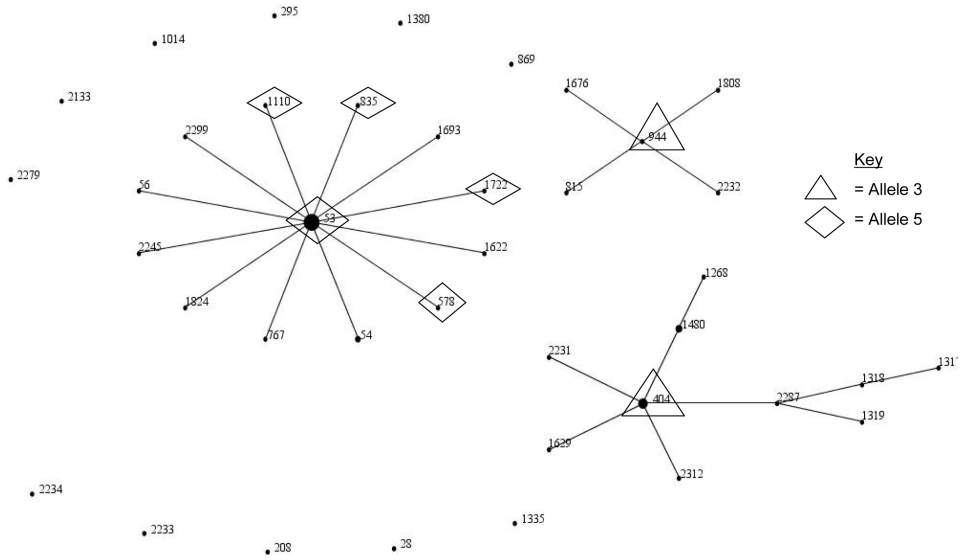

Figure 3. eBURST diagram showing the relationship between serotype 8 clones of *Streptococcus pneumoniae* as well as the distribution of pneumolysin alleles within the serotype 8 pneumococcal population.

prevalence in serotype 1–related IPD [17], which shows ongoing clonal expansion of ST306 to become the dominant clone in this serotype. These results, together with the observed geographical spread of ST53-related clones, allow us to hypothesize that allele 5 may have been present in ST53 before being acquired by ST306 pneumococci. Allele 5 may then facilitate clonal expansion of pneumococci. However, we appreciate that the nature of the MLST database means that internationally disseminated clones of the ST306 complex may exist.

There appears to be a relationship between the mutations carried by alleles 3, 5, and 10 and the electophoretic mobility of these proteins in SDS-PAGE. Proteins of these alleles have reduced mobility and so have an apparently higher molecular weight [16]. These alleles share the mutation A265S and the deletion of amino acids at positions 270 and 271. These may be responsible for the reduced mobility; however, allele 6 also possesses these mutations and shows no reduced mobility, although it has a further S167F mutation that may affect mobility (data not shown).

ST53 and ST306 are unrelated, sharing only 1 housekeeping allele *(spi)*. Furthermore, the sequences of the other housekeeping alleles are no more similar than those of 2 random clones (data not shown). This shows that there is no obvious relation between the 2 predicted founders of the clonal complexes associated with allele 5.

Y150H is the only mutation unique to the nonhemolytic allele 5. The tyrosine at this position was found to be fully conserved among all CDCs, whereas other positions of mutation in pneumolysin were shown to vary among CDCs (data not shown). This suggests an importance of this residue in the hemolytic activity of these toxins. Purified toxin possessing only the mutation Y150H has hemolytic activity of 478 HU/mg, implying that, although this mutation severely depletes hemolytic activity, the other mutations are also required to abrogate the activity of the toxin.

Unlike pneumococci of most other serotypes, serotype 1 pneumococci are known to be associated with outbreaks of pneumococcal disease. Serotype 8 pneumococci have also been responsible for such outbreaks [25, 40]. In the present study of 121 pneumococcal isolates, nonhemolytic pneumolysin variants were found only in serotypes that have been associated with outbreak disease. We propose that there is an association between nonhemolytic pneumolysin and pneumococcal serotypes that are able to cause outbreaks. Although hemolytic activity may not be required for IPD, other activities of the variant toxins (e.g., complement activation and inflammation via interaction with TLR4) may contribute to the pathogenesis of infection. Whether there is a causal link between expression of nonhemolytic toxin and outbreaks will require a much larger study comparing outbreak and nonoutbreak isolates. Given that outbreaks of pneumococcal disease remain rare, such a study may prove to be difficult.

In conclusion, pneumolysin has a level of variation higher than previously thought, and the lytic activity of the toxin is not always required for IPD. Furthermore, we report the presence of an insertion sequence that abrogates pneumolysin expression in a clinical isolate. These findings imply that the roles played by pneumolysin may vary in pneumococci of different genetic backgrounds. Nonhemolytic alleles appear to be confined to certain unrelated clonal complexes that have undergone expansion. We propose that this property of pneumolysin in certain genetic backgrounds can play a role in driving such

clonal expansion and may have an involvement in outbreaks of pneumococcal disease.

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